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STABILIZATION OF THE PHOSPHATE RATIO OF SEA WATER BY FREEZING

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ABSTRACT

In oceanographic investigation, it is often desirable to stabilize organic and certain inorganic components of sea water for delayed analysis. This stabilization, which cannot be achieved with chemical additives, is necessary because of biotic activity. Instability in the concentration of inorganic phosphorus is demonstrated in samples of sea water held at room temperature, while stability is demonstrated in samples quick-frozen and held at subzero temperatures.

Gains in productive manpower, in precision, and in the variety of analyses possible can all result from the adoption of this technique. Because the greater precision inherent in this procedure permitted more accurate analyses, it has already become evident that spatial variability of inorganic phosphorus in sea water is greater than has been recognized and should be investigated further.

STABILIZATION OF THE PHOSPHATE RATIO OF SEA WATER BY FREEZING

By Albert W. Collier, *Fishery Research Biologist*
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It is the accepted practice in oceanographic investigations to make all chemical analyses, except salinity determinations, at sea. This is necessary because there has been no way in which samples could be stabilized for the relatively long period intervening between the collection of samples and the return to base. Only a limited number of analyses can be performed at sea, as a rolling, vibrating ship is not suitable for the delicate instruments required to make many of the chemical analyses. The chemists assigned to oceanographic investigations have thus been forced to make determinations under conditions unfavorable to producing the best quality or the greatest quantity of work.

Since the program of the Gulf Fishery Investigations of the United States Fish and Wildlife Service necessitated expansion of chemical investigation into the fields of organic chemistry and of biochemistry, it was essential that we find some method of overcoming the difficulties to making chemical analyses at sea. We desired to increase the precision and quality of work and to take advantage of the gain in productive manpower inherent in chemists working ashore, so as to make an intensive study of the phosphorus economy of the sea, reexamine the nitrate problem, and study the organic compounds present in sea water.

This paper reports the first of a series of experiments to test the stability of certain organic and inorganic complexes in sea water after the samples have been quick-frozen. The analysis for inorganic phosphorus is universally used in oceanography and marine biology and is very sensitive to changes in biological activity. Accordingly, we selected it as the first of a series for testing and reporting.

TECHNIQUES OF FREEZING AND ANALYSIS

Sea water was collected from Offat's Bayou, a small inlet on the bay side of Galveston Island, and from the open beach of the Gulf of Mexico near the laboratory. Normally, the waters of Offat's Bayou are richer in phosphate than are the waters of the Gulf of Mexico. The bottles found most satisfactory for this work were 4-ounce prescription bottles with molded plastic screw caps. They withstood the freezing well with only occasional breakage.

Freezing was accomplished by immersing the sample bottles in a bath of dry ice and ethyl alcohol. Preliminary tests demonstrated that ordinary refrigeration did not stabilize the materials, and that even freezing in the ice-tray section of an ordinary refrigerator was too slow. The dry-ice-and-alcohol bath made an easily portable arrangement, and froze the samples quickly enough to be effective.

Conditions aboard ship made certain modifications of this method necessary. Samples to be frozen were collected in glass culture tubes (25 x 200 millimeters) fitted with molded plastic screw caps. To speed the freezing of sea-water samples and to save time on shipboard, the sample tubes were prerinsed three times with distilled water and thoroughly drained in the shore laboratory before the ship sailed. Before the samples were put in the freezing bath the tubes were tightly sealed at the junction of cap and tube with plastic insulating tape. This prevented contamination by the freezing solution or by the water of the bath during thawing.

Freezing was done in a vat of 38 percent ethylene glycol held at an average temperature of −5° F. in a commercial quick-freeze cabinet in-

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stalled for the purpose on the *Alaska*. The samples collected at one station were left in the freezing bath until the next station was reached, although they were well below 0° F. in less than 4 minutes. The samples were recorded according to their permanently affixed serial numbers.

The inorganic phosphorus was determined by the method described by Robinson and Thompson (1948) except that color intensity was measured with a Fisher AC electrophotometer. Our analyses were within the 10-percent limits of error given by Robinson and Thompson. All references to concentration of phosphorus are in terms of μ-gram atoms of phosphate phosphorus per liter.

EXPERIMENTS TESTING THE BEHAVIOR OF INORGANIC PHOSPHORUS IN SEA WATER

Experiment 1

Experiment 1 was set up to record any changes that occurred in the concentration of inorganic phosphorus in sea water left standing in sample bottles at room temperature. Such data were needed for comparison with those from the frozen samples.

Sea water was collected in Offat's Bayou and transported to the laboratory in a 5-gallon glass container. There it was filtered into a second glass container to remove gross particulate material, and from it 107 samples were siphoned into the prescription bottles. These samples were arranged in sets of 8, 9, or 10 bottles each, to be used as replicates for analysis at selected time intervals, and were placed on a table in the laboratory.

The initial phosphorus determinations were made after the water was filtered and before the first sample was drawn. The average value for each subsequent set of replicates was compared with the average of this initial set of eight. The sets comprising the series were analyzed consecutively at comparatively short intervals for the first 27 hours, and at longer intervals thereafter. The final analysis was made 29 days after the experiment was started. The results for the several time intervals, together with certain statistics pertaining to them, are shown in table 1.

The initial concentration was 2.54, but within 2 hours the next set gave a value of 2.58, to be followed by a drop to 2.45. This decline continued until the twenty-first hour when the concentration was 1.60. After this there were minor fluctuations around 2.00 for the remainder of the period. The "Student" t test was applied for comparing the mean of the initial set with the means of the subsequent sets, ending with the fifth hour.

The data for the first three time intervals were tested by the analysis of variance. The analysis was not extended beyond the fifth hour since the changes beyond that time were obviously significant. The means tested within the 5-hour period demonstrate heterogeneity, since F exceeds 32 which is significant at the 1-percent level.

TABLE 1.—*Changes in inorganic-phosphorus concentration of sea water occurring at room temperatures*

A. VALUES IN μ-GRAM ATOMS OF PO_4-P PER LITER

Time interval	Number in set	Mean	Standard deviation	Fiducial limits ($t_{.01}$)
Initial	8	2.54	0.092	0.11
2 hrs.	9	2.58	.067	.07
3 hrs.	10	2.45	.085	.08
5 hrs.	10	2.27	.048	.04
21 hrs.	10	1.60		
27 hrs.	10	1.68		
2 days	10	2.02		
3 days	10	2.20		
17 days	10	2.04		
21 days	10	1.93		
29 days	10	2.20		

B. COMPARISON OF MEAN OF INITIAL SET WITH MEANS OF SUBSEQUENT SETS

Time interval	Difference between means	Value of t	Degrees of freedom
2 hrs.	+0.04	1.045	
3 hrs.	−0.09	*2.097	
5 hrs.	−0.27	**7.986	

C. ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between sets	3	0.532	0.177
Within sets	33	.180	.005
Total	36	.712	

F=**32.24 between sets

Experiment 2

Experiment 2 was designed to test the rate of change of inorganic phosphorus at subzero temperatures. As in experiment 1, sea water was taken at Offat's Bayou and filtered at the laboratory to remove gross particulate material. Ten sets of samples were drawn, the first analyzed at once and the remaining nine frozen for later analysis. It required 15 minutes to draw and freeze each of the nine sets. The first was analyzed

on the following day, the last on the forty-fifth day. The results are shown in table 2.

While only small differences developed between the means of the initial and the final sets, five means were different, according to the t test. These variations between time intervals, while slight, are reflected in an F value of 3.78, which is significant at the 1-percent level.

From the results of these first two experiments it became suspect that the 2-hour interval required to draw and freeze the 10 sets introduced a variable, in that changes in inorganic-phosphorus content were taking place even in that short interval. Subsequent experiments were designed to control this variable.

TABLE 2.—*Changes in inorganic-phosphorus concentration of sea water occurring at subzero temperatures*

A. VALUES IN μ-GRAM ATOMS OF PO_4-P PER LITER

Time interval	Number in set	Mean	Standard deviation	Fiducial limits ($f_{.01}$)
Initial	10	2.05	0.097	0.100
1 day	10	1.72	.181	.186
2 days	10	1.95	.097	.100
3 days	10	1.92	.225	.231
6 days	7	1.84	.172	.241
8 days	10	1.88	.162	.166
21 days	8	1.90	.141	.175
29 days	10	1.89	.090	.090
37 days	9	2.02	.140	.156
45 days	8	1.99	.155	.192

B. COMPARISON OF MEAN OF INITIAL SET WITH MEANS OF SUBSEQUENT SETS

Time interval	Difference between means	Value of t	Degrees of freedom
1 day	−0.33	**5.072	18
2 days	−.10	2.301	18
3 days	−.13	1.677	18
6 days	−.21	**3.179	15
8 days	−.17	*2.847	18
21 days	−.15	*2.667	16
29 days	−.16	**3.868	18
37 days	−.03	.509	17
45 days	−.06	1.046	16

C. ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between sets	9	0.777	0.086
Within sets	82	1.868	.023
Total	91	2.646	

F=**3.78 between sets

Experiment 3

Experiment 3 was designed, as were experiments 1 and 2, to measure the differences between rates of change in inorganic phosphorus stored at room temperature and at subzero temperatures. It was modified from these by the addition of inorganic and organic phosphorus, the former as potassium phosphate (dihydrogen) C. P., the latter as sodium glycerophosphate (Eastman). These were added to ensure a sufficient substrate of phosphorus to permit greater changes in concentration than could occur in phosphorus-deficient natural sea water.

The sea water for this experiment was taken from Galveston Beach. After adding the phosphorus, the mixture was filtered before drawing the samples. Altogether, 13 sets of samples were drawn, 5 to be kept at room temperature, 8 to be frozen. The results of these experiments are shown in tables 3 and 4.

In the 25 days during which the samples were held at room temperature, the concentration of inorganic phosphorus nearly doubled (table 3). As shown by the t test, each set of samples was significantly different from the initial set. The high values for the mean squares in the analysis of variance indicate the great differences which resulted between sets as compared with the differences within sets.

TABLE 3.—*Changes in inorganic-phosphorus concentration of sea water with added phosphorus, occurring at room temperature*

A. VALUES IN μ-GRAM ATOMS OF PO_4-P PER LITER

Time interval	Number in set	Mean	Standard deviation	Fiducial limits ($f_{.01}$)
Initial	19	5.83	0.1202	0.134
1 day	10	6.38	.148	.152
3 days	10	8.01	.357	.367
17 days	10	8.90	.226	.232
25 days	10	10.24	.232	.238

B. COMPARISON OF MEAN OF INITIAL SET WITH MEANS OF SUBSEQUENT SETS

Time interval	Difference between means	Value of t	Degrees of freedom
1 day	0.55	**11.193	27
3 days	2.18	**24.672	27
17 days	3.07	**48.975	27
25 days	4.41	**69.141	27

C. ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between sets	4	162.837	40.709
Within sets	54	2.526	.047
Total	58	165.362	

F=**869.85 between sets

By comparison, and in confirmation of the results shown in experiment 2, while the mean of the set held 61 days at subzero temperatures is not significantly different from that of the initial set, 4 of the 7 sets show differences with the t test (table 4). The analysis of variance also shows a possible significant difference between sets.

From these results it became increasingly evident that the time required to process the samples was introducing an uncontrolled variable, with changes in the ratio of inorganic to organic phosphorus being more rapid than had been suspected. The following experiment was designed to control this.

TABLE 4.—*Changes in inorganic-phosphorus concentration of sea water with added phosphorus, occurring at subzero temperatures*

A. VALUES IN μ-GRAM ATOMS OF PO_4-P PER LITER

Time interval	Number in set	Mean	Standard deviation	Fiducial limits ($t_{.01}$)
Initial	19	5.83	0.115	0.076
1 day	9	5.64	.219	.244
2 days	10	5.54	.268	.275
3 days	10	5.64	.334	.343
10 days	10	5.63	.400	.411
13 days	10	5.78	.187	.193
18 days	9	5.58	.156	.175
61 days	6	5.78	.172	.284

B. COMPARISON OF MEAN OF INITIAL SET WITH MEANS OF SUBSEQUENT SETS

Time interval	Difference between means	Value of t	Degrees of freedom
1 day	−0.19	**2.914	26
2 days	−.29	**4.057	27
3 days	−.19	*2.225	27
10 days	−.20	.016	27
13 days	−.05	.828	27
18 days	−.25	**4.762	26
61 days	−.05	.710	23

C. ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between sets	7	0.904	0.129
Within sets	75	4.368	.058
Total	82	5.272	

F=*2.220 between sets

Experiment 4

Experiment 4 was designed to appraise the effects of the delay inherent in drawing a large number of samples on the concentration of inorganic phosphorus. In this experiment, six sets of samples were drawn as rapidly as possible directly from Offat's Bayou. Filtering was eliminated to expedite the operation. In the initial set, the molybdate reagent was added to stabilize the samples, and the set was analyzed immediately on return to the laboratory. Subsequent samples, held at room temperature, were analyzed at the intervals shown in table 5.

The increases of 0.2 μ-gram atoms from 1.38 during the first 30 minutes and of 0.36 μ-gram atoms within the first 90 minutes, and the subsequent rise to 1.80 and decline to 1.68 μ-gram atoms, indicate how rapidly changes can take place at room temperature and explain, in part at least, the variations which developed in experiments 2 and 3. It is important to note that, while each set of samples was significantly different from the initial, as shown by the t test, and that the variations between sets in the analysis of variance was large in relation to the variance within sets, the standard deviations and the variance within sets were nominal. The importance of adequate stabilization or immediate analysis in determining the inorganic-phosphate ratio is here clearly demonstrated.

TABLE 5.—*Changes in inorganic-phosphorus concentration of sea water occurring at room temperature*

A. VALUES IN μ-GRAM ATOMS OF PO_4-P PER LITER

Time interval	Number in set	Mean	Standard deviation	Fiducial limits ($t_{.01}$)
Initial	9	1.38	0.067	0.075
30 min	5	1.60	.122	.252
90 min	7	1.74	.098	.137
3 hrs	8	1.80	.160	.198
4 hrs	9	1.78	.083	.093
5 hrs	9	1.68	.097	.109

B. COMPARISON OF MEAN OF INITIAL SET WITH MEANS OF SUBSEQUENT SETS

Time interval	Difference between means	Value of t	Degrees of freedom
30 min	0.22	**4.422	12
90 min	.36	**8.774	14
3 hrs	.42	**7.209	15
4 hrs	.40	**11.239	16
5 hrs	.30	**7.635	16

C. ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between sets	5	1.067	0.213
Within sets	41	.464	.011
Total	46	1.531	

F=**18.85 between sets

Experiments 5 and 6

Experiment 5 was designed to obtain more nearly exact information on the effect of freezing on the changes in the concentration of inorganic phosphorus by minimizing the delay inherent in drawing and freezing a large number of samples. As in experiment 4, samples were drawn from Offat's Bayou as rapidly as possible and without filtering. The samples were frozen immediately. Again, the molybdate reagent was added to the initial set, which was analyzed immediately on return to the laboratory. In each, the molybdate reagent was added to the sample as it was placed in the water bath so as to minimize changes which might take place in the period of thawing.

In this experiment, the first set of samples was analyzed 90 minutes after freezing, the last set 4¼ hours later (table 6). With this technique, no differences are evident either in the t test or in the F value of the analysis of variance.

TABLE 6.—*Changes in inorganic-phosphorus concentration of sea water occurring at subzero temperatures*

A. VALUES IN μ-GRAM ATOMS OF PO_4-P PER LITER

Time interval	Number in set	Mean	Standard deviation	Fiducial limits ($t_{.01}$)
Initial	9	2.90	0.492	0.551
1¼ hrs	8	2.65	.093	.114
3¼ hrs	9	2.70	.206	.231
4¼ hrs	10	2.95	.530	.544
5¼ hrs	11	2.80	.417	.399

B. COMPARISON OF MEAN OF INITIAL SET WITH MEANS OF SUBSEQUENT SETS

Time interval	Difference between means	Value of t	Degrees of freedom
1¼ hrs	−0.25	1.409	15
3¼ hrs	−.20	1.124	16
4¼ hrs	+.05	.212	17
5¼ hrs	−.10	.492	18

C. ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between sets	4	0.583	0.146
Within sets	42	6.605	.157
Total	46	7.188	

$F < 1$ between sets

The same techniques were employed in experiment 6 as in experiment 5, but the samples were held for a longer period of time, as shown in table 7. Here, again, the rapid freezing techniques minimized changes during the period of collection, so that no significant changes in the concentrations of inorganic phosphorus were evidenced in the t test. The difference shown in the F test of the analysis of variance, being greater within than between sets, is probably due to particulate material, since these samples were not filtered. The variance between sets is at a minimum. From these experiments, it is apparent that rapid freezing and holding of samples at subzero temperatures effectively stabilizes the inorganic-phosphate ratio, so permits of analysis even weeks after collection with greater assurance of accuracy than if any delay is encountered between collection and analysis.

TABLE 7.—*Changes in inorganic-phosphorus concentration of sea water occurring at subzero temperatures*

A. VALUES IN μ-GRAM ATOMS OF PO_4-P PER LITER

Time interval	Number in set	Mean	Standard deviation	Fiducial limits ($t_{.01}$)
Initial	7	2.26	0.127	0.178
2 hrs	10	2.24	.196	.201
3¼ hrs	7	2.29	.069	.097
1 day	10	2.24	.237	.243
3 days	10	2.27	.275	.283
7 days	9	2.27	.206	.231

B. COMPARISON OF MEAN OF INITIAL SET WITH MEANS OF SUBSEQUENT SETS

Time interval	Difference between means	Value of t	Degrees of freedom
2 hrs	−0.02	0.202	15
3¼ hrs	+.03	.527	12
1 day	−.02	.173	15
3 days	+.01	.115	15
7 days	+.01	.108	14

C. ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between sets	5	0.014	0.003
Within sets	47	1.995	.042
Total	52	2.009	

$F < 1$ between sets

CONCLUSIONS

Robinson and Thompson (1948) stated that determination of the inorganic-phosphorus content of sea water must be made as soon as possible after collection of the samples. This is borne out by the results of this study which give definite proof that changes can take place within 30 minutes. The data shown in table 5 demonstrate this.

It is also clearly demonstrated that these same changes can be completely arrested for prolong periods by quick-freezing, and that this method stabilization has sufficient reliability for gener adoption.

LITERATURE CITED

ROBINSON, REX J., and THOMAS G. THOMPSON. 1948. The determination of phosphates in s water. Jour. Marine Research 7 : 33–41.

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